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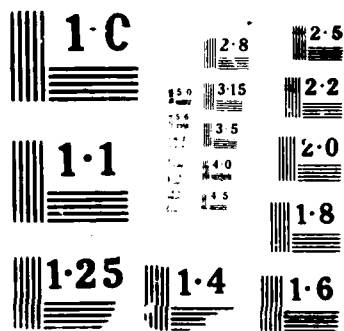
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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The role of brain cholinergic receptors in regulation of physiological and behavioral function was examined by inducing a change in the number of muscarinic cholinergic receptors and studying the effects of these changes on tolerance development and spatial learning in inbred mice. Chronic treatment with oxotremorine caused a down-regulation of receptors in five brain regions including hippocampus and cortex, regions thought to mediate spatial learning. The time course of receptor recovery was compared to the loss of tolerance and changes in spatial learning ability. While receptor numbers were decreased for as long as 4 days after cessation of treatment, tolerance to oxotremorine was only detected for 48 hrs after removal from chronic treatment. Likewise, impairment of spatial learning was evident when animals began training at 24 hrs.			
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19. after cessation of treatment but learning was normal by 48 hrs after treatment. These results indicate that the status of cortical and hippocampal cholinergic function may be important for initial behavioral effects, but that there must be other factors in addition to receptor number that are important in the regulation of physiological and behavioral function.

The possible roles of other cholinergic markers and other neurotransmitter systems are currently being investigated including NMDA receptor function and phosphorylation of hippocampal proteins. *Keywords: Learning, Brain, nerve transmission*

ANNUAL TECHNICAL REPORT FOR 1986-1987

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Principle Investigator: Jeanne M. Wehner

CO-Principle Investigator: Allan C. Collins

Summary

The ongoing focus of our research is to determine the role neurotransmitter receptor regulation in the mediation of behavioral and physiological function. The mouse has been used as a model system in this work because inbred strains exist that differ in certain behaviors and these can be used as tools to examine mechanistic questions. Spatial learning as examined by the Morris water test and tolerance development as examined by body temperature depression and rotarod performance were used as the behavioral and physiological measures that might reflect altered CNS function in mice as a result of alterations in neurotransmitter receptors by chronic exposure to cholinergic drugs.

C57BL/6Ibg mice perform well in the Morris water test indicating that they acquire and retain spatial learning while DBA/2Ibg mice do not. Previous studies using chronic treatment with diisopropylfluorophosphate (DFP) impaired acquisition of spatial learning in C57BL mice, but produced no effect in DBA mice indicating that DBA mice do not use a cholinergic form of learning to attempt the spatial learning task. The data also suggest a role of muscarinic cholinergic receptors in the acquisition of spatial learning since the number of these receptors is reduced in cortex and hippocampus in the impaired animals.

The role of cortical and hippocampal muscarinic receptors in spatial learning was examined in more detail in C57BL mice by chronically treating animals with the agonist, oxotremorine. This agent produced a reduction in muscarinic receptors in cortex and hippocampus and receptors did not return to control levels for at least 4-8 days after cessation of treatment. Tolerance to oxotremorine developed as a result of chronic treatment. At 24 hrs after treatment, impairment of the acquisition of spatial learning was observed. However, although receptors were reduced, tolerance to the effects of a challenge dose of oxotremorine was lost by 48 hrs after cessation of treatment. Likewise, animals that began acquisition training in the spatial learning test at 48 hrs after treatment were devoid of impairment. These results indicate 1) that the status of the hippocampal &/or cortical cholinergic system is important during initial acquisition of learning and 2) there is a dissociation between receptors and the duration of impairment. Other cholinergic markers and mechanisms are currently being investigated including high affinity choline uptake. Because of the involvement of the neurotransmitter glutamate in learning, the hippocampal glutaminergic system has been investigated in C57 and DBA mice. The phosphorylation of the hippocampal F1 protein which has been shown to be correlated with the process of Long Term Potentiation is also under investigation.

2) Research objectives and status of the research

The objectives of the second year of work were to a) examine the effects of down-regulation of muscarinic receptors on tolerance development and spatial learning; b) to derive the appropriate conditions to up-regulate brain muscarinic cholinergic receptors; c) to investigate further the genetic regulation of spatial learning; d) to initiate a series of investigations of other cholinergic markers that might be related to spatial learning; and e) to begin investigations of noncholinergic markers in spatial learning.

The status of the research addressing these goals is described below:

a) Down-regulation of muscarinic receptors in C57BL.

Although we had demonstrated that DFP could down-regulate muscarinic receptors and produce an impairment of spatial learning, several questions prompted us to pursue another form of drug-induced down-regulation of receptors. For example, DFP produces a loss of both muscarinic and nicotinic receptors but it does not induce a clearly defined tolerance to DFP. Moreover, it is an organophosphate and may produce some neurotoxic side-effects. We therefore used the muscarinic agonist, oxotremorine to produce a down-regulation of muscarinic receptors. Since tolerance to oxotremorine results from chronic treatment, we had a second set of measures that could be used in addition to spatial learning. To examine the effects of chronic oxotremorine infusion, we also examined the return of receptors, the loss of tolerance, and the recovery of spatial learning. The results have been very informative in regards to the role of receptors in regulating behavioral and physiological function.

In figure 1 is shown the loss and recovery of muscarinic receptors after 6 days of chronic infusion of oxotremorine through a subcutaneous cannula. The doses of oxotremorine were increased gradually from 1-5 mg/kg/hr during the four days preceeding the final 5 mg/kg/hr dose. Muscarinic receptors were measured by ³H-QNB binding. A significant decrease in receptors was observed in all brain regions except striatum. The recovery rate of receptors to normal levels was dependent on the brain region, but both cortical and hippocampal receptors had returned to normal by 4 days after cessation of treatment. This meant that we were able to conduct our three days of spatial learning behavioral testing at a time period when receptors were reduced in the two areas of importance, cortex and hippocampus.

Mice that had been infused with oxotremorine or saline were tested for the development of tolerance to a challenge of oxotremorine at various days after cessation of infusion. Two measures of tolerance were used, depression of body temperature and rotarod performance. After extensive work, we determined that body temperature was a better measure of tolerance because it was less variable. In figure 2 is shown the dose response curves for control (saline-infused) and oxotremorine infused at 0-8 days of withdrawal as measured by depression of body temperature.

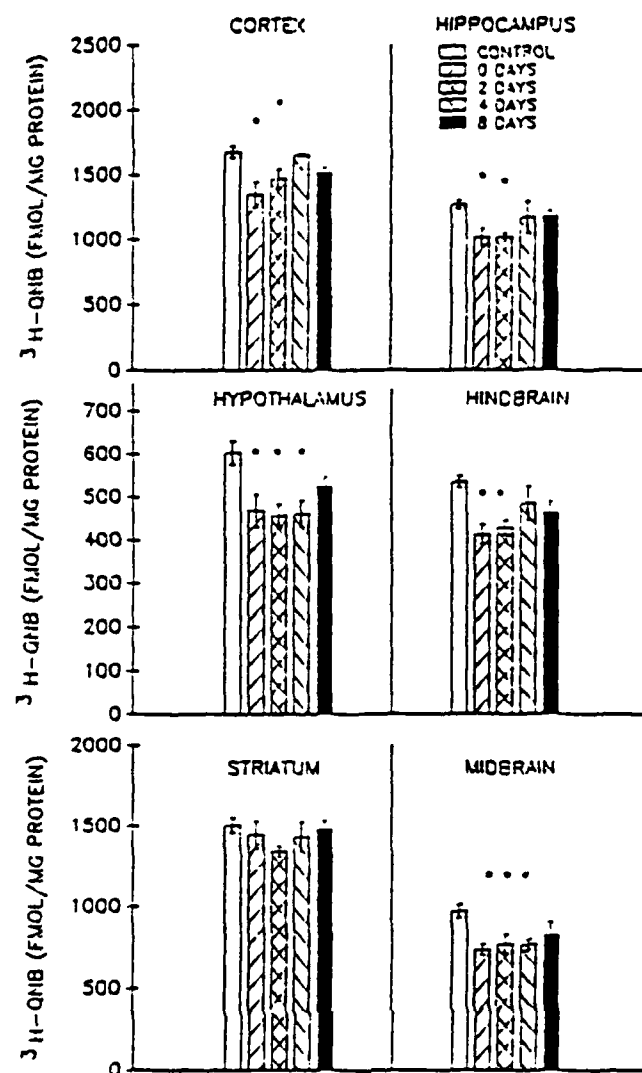


FIGURE 1

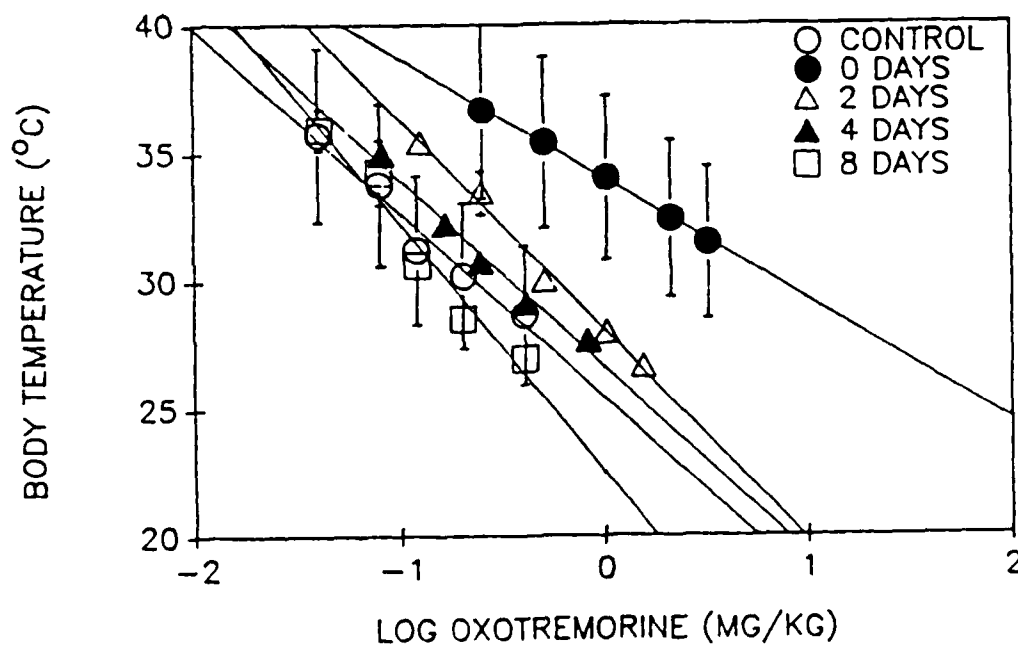


FIGURE 2

Figure 3 shows the data for tolerance loss as measured by rotarod performance. Figure 4 A shows a representation of the dose to produce a body temperature of 35°C (ED₃₅) as a function of days after withdrawal. Figure 4B shows the same representation for rotarod performance as shown by an ED₅₀. These data indicate that significant tolerance develops to oxotremorine but that the degree of tolerance is no longer significant by 48hrs after removal from infusion. This means that loss of tolerance can be dissociated from muscarinic receptor changes in every brain region measured. Thus, although constant agonist stimulation of the receptor no doubt has a role in tolerance development, something more is occurring in the CNS to mediate these changes.

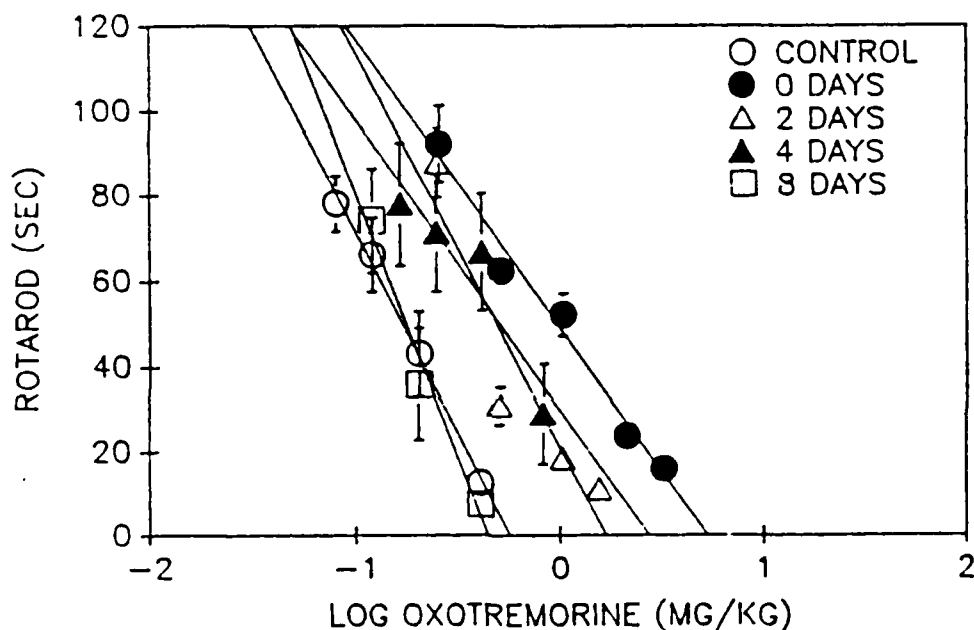


FIGURE 3

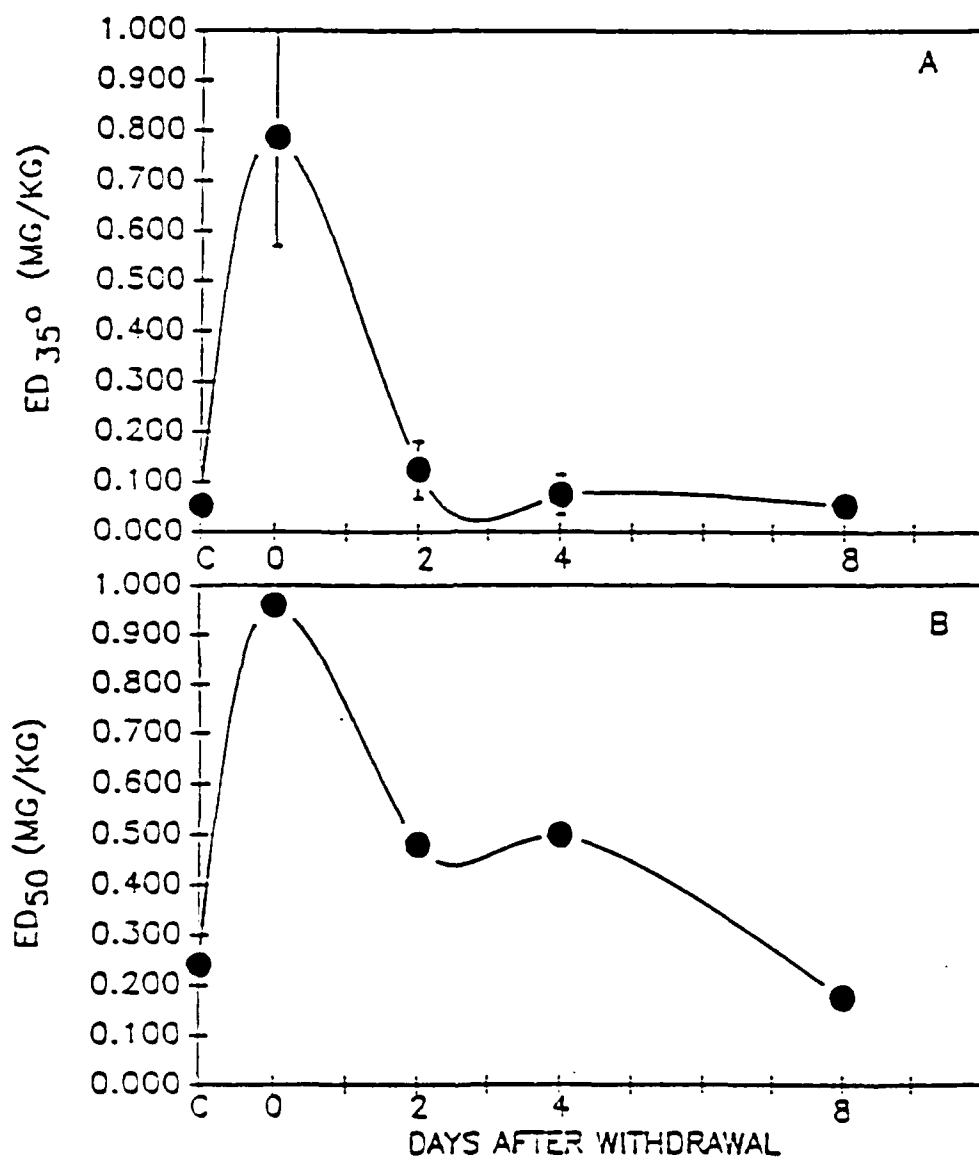


FIGURE 4

The effects of chronic oxotremorine treatment on spatial learning also showed a similar picture. Mice were trained in the Morris swim test and the degree of impairment in acquisition of spatial learning was assessed using a probe trial test. The hidden platform is removed from the pool and the mice are videotaped while they examine the pool area. Those mice that are able to perform the test will cross the site of training more often than the other three sites and will spend a larger proportion of their total test time in the quadrant that held the trained platform site. When the animals began acquisition training at 24 hrs after cessation of oxotremorine treatment, oxotremorine-treated animals perform poorer than the saline-treated controls in the probe trial test which is performed on the third day of training. However, animals that were trained beginning at 48 hrs after cessation of treatment, were no longer impaired and performed like saline-treated mice in the probe trial test.

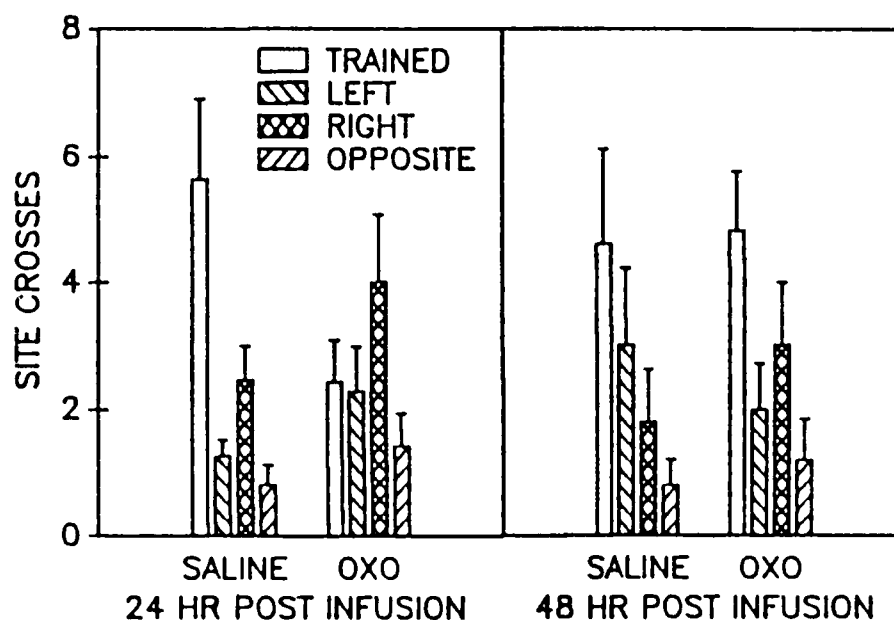


FIGURE 5

As with the loss of tolerance, the impairment of spatial learning produced by oxotremorine treatment is short-lived. This impairment is unlike that produced by DFP treatment where mice were impaired for as long as 16 days post-treatment. We conclude from these studies 1) that the status of the cholinergic system is important during initial acquisition and 2) tolerance development and spatial learning involves more than the number of cortical and hippocampal muscarinic receptors.

At this time we are not certain what those factors might be but must consider further other subtypes of cholinergic receptors as well as changes in coupling mechanisms. Because DFP produces a loss in both nicotinic and muscarinic receptors, we will be performing chronic treatments in mice with nicotine. This treatment produces an up-regulation of nicotinic receptors so that we might predict that if nicotinic receptors are involved in spatial learning that spatial learning ability might improve after chronic treatment.

Dr. Maier did perform a small experiment in rats that had been injected with nicotine and saw no affect of nicotine, but the number of rats were small and the results questionable.

The question of muscarinic receptor subtypes may also be important. Specifically, oxotremorine is a full agonist at M2 receptors but not very potent at M1 receptors. Therefore, treatment with a more selective M1 agent would be helpful. This is difficult because there are few drugs of this character available. Pirenzepine is a selective (but not specific antagonist) of M1 receptors; however, it does not cross the blood brain barrier in sufficient quantities to induce an up-regulation of receptors. Implantation of cannula for direct CNS administration is difficult in mice so that before embarking on such experiments we have chosen to first up-regulate muscarinic receptors as a consequence of treatment with the nonspecific cholinergic antagonist, scopolamine. If we do observe a difference then it may be worth the effort to test the effects of a more selective antagonist.

b.) Up-regulation of muscarinic receptors

Up-regulation of muscarinic receptors is more difficult than down-regulation. There are few antagonists that can pass the blood brain barrier so that chronic treatment is difficult but scopolamine can be administered in periphery to induce CNS changes. Unfortunately, it has a biphasic dose response curve for receptor up-regulation and it is difficult to monitor mice on the drug because they seem to die for unexplained reasons. We have been able to demonstrate that at 0.6 mg/kg/hr for 6 days C57BL mice show a significant up-regulation of ^3H -QNB binding in cortex and hippocampus as shown in figure 6. This up-regulation is still apparent at 18 hrs after removal from the scopolamine but is not significant in the hippocampus by 48 hrs after removal. Scopolamine-treated mice become supersensitive to the effects of oxotremorine as would be predicted if they have more muscarinic receptors. We do not know whether scopolamine treatment changes spatial learning because we have not tested an adequate number of mice to make a conclusion at this time. Our study is being conducted like the oxotremorine one described above. Animals will be subjected to acquisition training at an early time when receptors are in the up-regulated state, and then at a later time when the receptors are no longer up-regulated. This design should provide us with information on alterations in the status of the cholinergic system during acquisition.

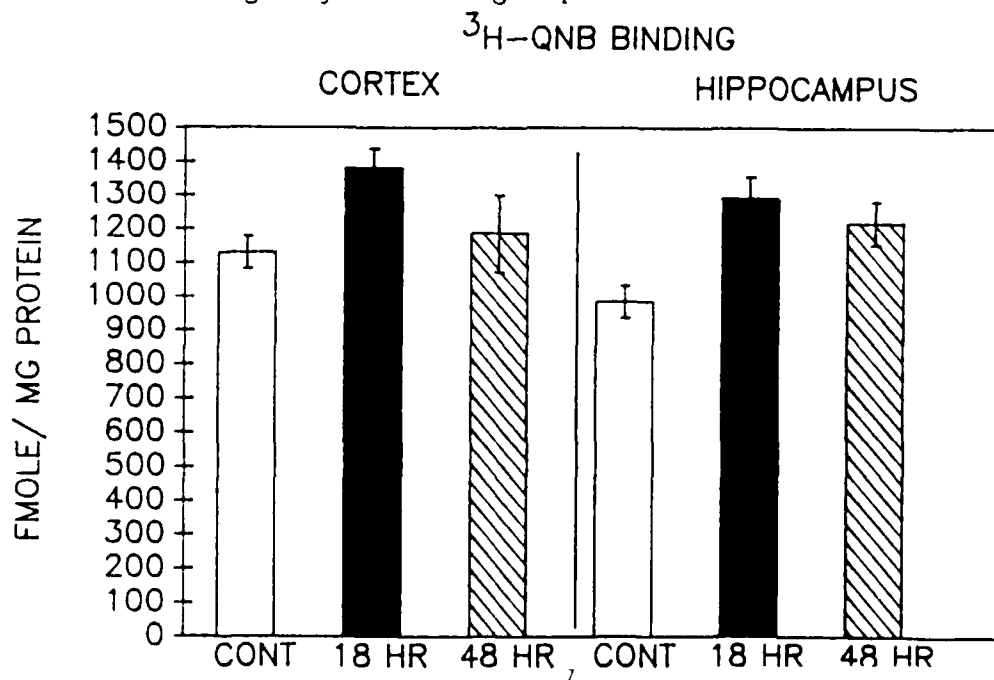


FIGURE 6

c) Genetic regulation of spatial learning

Having two inbred strains of mice that differ in spatial learning ability allows one to examine the genetic regulation of this behavior. We have almost completed a classical genetic analysis of spatial learning using crosses of the good learners, C57BL mice with the poor learners, DBA mice. In order to analyze the data resulting from such crosses we must derive one score that represents the ability level of the individual mice. For this purpose we have used a preference score which is defined as the number of correct crosses at the trained site minus the mean of the incorrect crosses at other sites. In figure 7 is shown the results of these crosses. It can be seen that the mean preference scores for each genetic stock of mice are better than that of either parent. This phenomenon is called hybrid vigor, heterosis or overdominance. It can be interpreted using quantitative genetic methods to support the idea that genes for spatial learning are inherited in a dominant fashion. But what is also clear is that both DBA and C57 mice possess some genes that lead to increased spatial learning and overall fitness. Such a pattern is expected when the trait is of evolutionary significance as spatial learning might be in the wild.

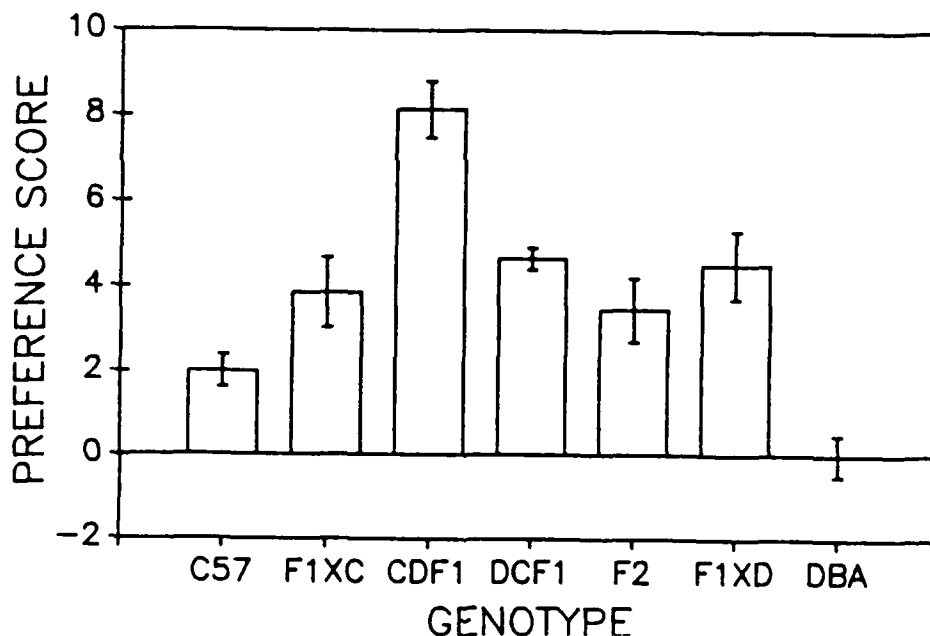


FIGURE 7

In addition to the basic information that a classical analysis can provide, these genetic stocks of mice can be used to answer mechanistic questions. Although not evident when in a population mean such as that shown in figure 7, some of these generations of mice show segregated pattern of spatial learning. For example, in the F₂ generation (F₁ X F₁) there is a gradation of learning ability because of the mixture of the genes from the two parental stocks. This is also true of the backcross generations (F₁ X C57 or F₁ X DBA). Those mice exhibiting a gradation should show a gradation in a biochemical or electrophysiological parameter that mediates spatial learning. We have been maintaining these different stocks of mice and have been using them for some mechanistic studies described below.

d.) Other cholinergic markers and spatial learning

It is obvious from the literature and our studies that muscarinic receptor number is not the sole determinant of spatial learning ability in inbred mice. The differences that we observe in the degree of impairment after DFP versus oxotremorine treatment would indicate that DFP may be producing a more dramatic alteration. This is despite the fact that the loss of muscarinic receptors is approximately the same after both treatments. Within the cholinergic system, other enzymes or uptake systems could be important in the regulation of spatial learning and could be affected by chronic treatment. Such candidates are choline acetyltransferase, acetylcholinesterase, and high affinity choline uptake. The results of work from Allan Collins and his colleagues obtained during a previously funded AFOSR project allowed us to dismiss several of these possibilities. Choline acetyltransferase is not altered as a result of oxotremorine treatment so it is unlikely to mediate the effects we have observed after chronic oxotremorine treatment. Moreover, C57 and DBA mice do not differ in this activity. Likewise, the ability of DFP to inhibit acetylcholinesterase activity is not different in C57 and DBA mice eliminating it as a candidate for the difference between C57 and DBA mice.

High affinity choline uptake has not been measured in inbred strains. It does not change after chronic oxotremorine treatment, but it was not measured after chronic DFP treatment in mice. Thus we felt that we must examine choline uptake in both C57BL and DBA mice and also in C57BL mice after DFP treatment.

High affinity choline uptake was examined in cortex and hippocampus of C57BL and DBA mice. The K_m for choline was determined in cortex only because of the availability of tissue. Table 1 shows the results of this comparison.

TABLE I

CORTEX

	V_{\max}	K_d
C57BL	10.3 +/- 1.5 pmol/mg/min	1.01 +/- .24 μ M
DBA	8.9 +/- 1.1 pmol/mg/min	0.93 +/- .18 μ M

HIPPOCAMPUS*

C57BL	4.16 +/- .54 pmol/mg protein/ min
DBA	3.21 +/- .29 pmol/mg protein/ min

* performed at [choline] of 1.0 μ M

These data indicate that there are no strain differences in high affinity choline uptake. We are performing one more series of experiments on high affinity choline uptake in order to determine whether this cholinergic marker changes as a function of learning in C57BL mice and in F₂ and backcross genetic stocks generated as described above. It is possible that in C57 mice and relatives of C57, a variation in choline transport might be relevant since we are fairly confident that these strains use a cholinergically based form of spatial learning. Preliminary data indicate a significant correlation between choline uptake and the ability of the mice to learn.

Moreover, preliminary data indicate that DFP may change choline uptake in cortex, but we have not observed such a change in hippocampus. At this point it is too early to implicate choline uptake as an important biochemical mediator of any of the changes we have observed in spatial learning ability.

e.) Noncholinergic markers that may be involved in spatial learning.

Numerous studies on spatial learning and other forms of learning implicate the excitatory neurotransmitter, glutamate in the mediation of learning. These studies include both human studies indicating a loss of the NMDA receptors in Alzheimer's Disease and the results of animal studies which demonstrate that chronic pretreatment with the NMDA antagonist AP-5 prevents acquisition of spatial learning and the induction of Long Term Potentiation (LTP) in the hippocampus. For these reasons, we have initiated a series of studies on NMDA receptor function in the dentate gyrus.

The studies of the NMDA receptors in the dentate gyrus have been performed as an electrophysiological study. NMDA receptors are somewhat difficult to study devoid of activity from other glutamate subtypes, but we have analyzed them in a hippocampal slice preparation using extracellular techniques. Mg²⁺ is added to the media and after a period of time, population spikes are altered and epileptiform activity is observed (a series of additional population spikes).

When the antagonist AP-5 is added to the bath some of the spiking activity disappears because of the blockade of NMDA receptors. A cumulative dose-response curve can be generated by additional additions of AP-5.

We performed this type of analysis on C57 and DBA mice and observed that C57 mice were slightly more sensitive to the actions of AP-5. This difference was significant at the $P = .05$ level. Analysis of the other remaining subtypes of glutamate receptors using other pharmacological agents indicated no difference in the strains. We believe that this comparison needs to be extended to some of the other genetic stocks of mice and that it should be supplemented with some other evidence before we feel confident that this small difference is important.

The first strategy that we have begun is to examine the NMDA receptor system further is LTP in the two strains. We have purchased the necessary equipment for another research project so that this technology has been set up in our laboratories. In addition to baseline strain differences, we hope to evaluate the effects of in vivo treatment with glutaminergic and cholinergic drugs on LTP.

The second strategy will require a longer period of time and will be outlined in our renewal application. We intent to quantitate NMDA receptors in the hippocampus by receptor autoradiography and determine whether these electrophysiological changes are mediated by differing numbers of NMDA receptors in C57 and DBA mice.

In relation to LTP and the glutamate system, we have almost completed a study on hippocampal phosphorylation of the F1 protein. Routtenberg and his associates have promoted the idea that LTP is accompanied by an increase in the phosphorylation of a 47K protein in dorsal hippocampus. We have used their methods to analyze dorsal hippocampal protein phosphorylation in our genetic stocks to ask whether this mechanistic difference is correlated to spatial learning ability. In figure 8 is shown the data plotted as total latency score versus percent of phosphorylation of F1. Each circle on the graph represents an animal from the segregating generations (F_2 or backcrosses) of a cross of C57 with DBA. Although these animals reach criteria in the spatial learning test, they do exhibit gradations in their learning ability as seen by their cumulative scores. The correlation for these two parameters is $-.42$ and it is significant at the $P = .02$.

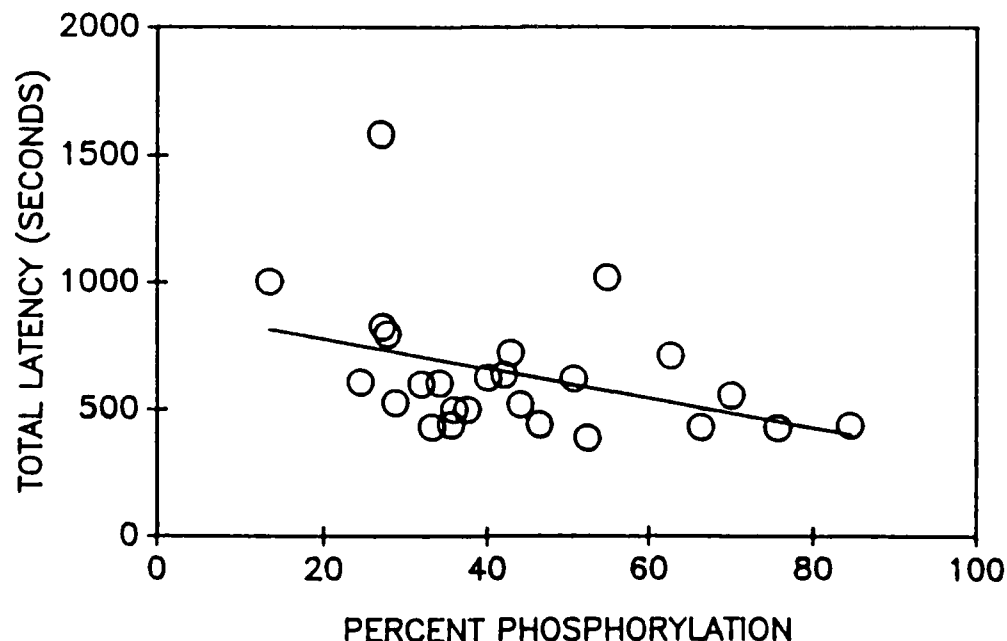


FIGURE 8

We are continuing to fill in more groups of animals and analyzing trained versus untrained mice before this work will be submitted for publication. We do believe on the basis of these data that Routtenberg's hypothesis that alterations in the Fl protein in the dorsal hippocampus as associated with learning is correct. We did not observe this change in four other proteins in the molecular weight range of 45-50.

In summary, our results on manipulation of the muscarinic cholinergic receptor system has lead us to consider a variety of mechanisms that might participate in regulation of tolerance development and spatial learning. Although we feel confident that the status of these receptors is important in behavioral and physiological functions, the regulation is more complex. We are therefore examining the involvement of other neurotransmitter systems, but know that in the future we must also examine the role of coupling mechanisms in the cholinergic system to understand better the consequences of these changes.

3) Publications and submissions for 1986-1987

1. Previously listed as submitted that are now published

Marks MJ, Stitzel JA, Romm E, Wehner JM, Collins AC: Nicotinic binding sites in rat and mouse brain: Comparison of acetylcholine, nicotine, and alpha-bungarotoxin. Molecular Pharmacology 30: 427-436, 1986.

Wehner JM, Murphy-Erdosh C, Smolen A, Smolen TN: Genetic variation in paraoxonase activity and sensitivity to diisopropylphosphofluoridate in inbred mice. Pharmacology Biochemistry and Behavior 28: 317-320, 1987.

Upchurch M, Wehner JM: Effects of chronic diisopropylfluorophosphate treatment on spatial learning in mice. Pharmacology Biochemistry and Behavior 27: 143-151, 1987.

2. In press or submitted

Upchurch M, Wehner JM: DBA/2Ibg mice are incapable of cholinergically-based learning in the Morris water task. Pharmacology Biochemistry and Behavior, in press.

Upchurch M, Wehner JM: Differences between inbred strains of mice in Morris water maze performance. Behavior Genetics, in press.

Wehner JM, Upchurch M: The effects of chronic oxotremorine treatment on spatial learning and tolerance development in mice. Pharmacology Biochemistry and Behavior, accepted with minor revisions, Oct. 10, 1987.

Collins AC, Romm E, Wehner JM: Nicotine tolerance: An analysis of the time course of its development and loss in the rat. Psychopharmacology, submitted.

3. In preparation

Collins AC, Romm E, Wehner JM: Nicotine tolerance and loss after chronic infusion in the rat. J.P.E.T. to be submitted in November, 1987

Wehner JM, Sleight S, and Upchurch M: Variable Phosphorylation of F1 protein as a function of spatial learning ability in genetic crosses of C57 and DBA mice. Brain Research to be submitted in December, 1987.

Upchurch M and Wehner JM: Classical genetic analysis of spatial learning in C57 and DBA mice. Beh. Genetics to be submitted in December, 1987.

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